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(54) Title: HLA-RESTRICTED HEPATITIS B VIRUS CTL EPITOPES

(57) Abstract

Cytotoxic T lymphocyte-stimulating peptides induce HLA-restricted responses to hepatitis B virus antigens. The peptides, derived from CTL epitopic regions of both HBV surface and nucleocapsid antigens, are particularly useful in the treatment and prevention of HBV infection, including the treatment of chronically infected HBV carriers. The peptides can be formulated as HBV vaccines and pharmaceutical compositions, such as lipid-containing compositions for enhancing the HLA-restricted CTL responses. The peptides are also useful in diagnostic methods, such as predicting which HBV-infected individuals are prone to developing chronic infection.

HLA-RESTRICTED HEPATITIS B VIRUS CTL EPITOPES

Related Applications

The present application is a continuation-in-part of U.S. Serial No. 07/874,491, filed April 27, 1992, which is a continuation-in-part of U.S. Serial No. 07/827,682, filed January 29, 1992, which is a continuation-in-part of USSN 07/749,568, filed August 26, 1991, each of which is incorporated herein by reference.

Background of the Invention

The hepatitis B virus (HBV) is not believed to be directly responsible for damage to hepatocytes, despite its predilection for infecting such cells. Rather, non-viral host factors are implicated in the pathogenesis of hepatitis. It is suspected that a variation in immune responsiveness to HBV infection may account for the wide diversity of syndromes associated with HBV infection.

Following an acute HBV infection, approximately 90% of affected adults recover without sequelae and develop immunity to the virus, although the clinical course of the infection during the acute phase can itself be quite variable. In 5-10% of infected adults, a chronic HBV infection becomes established. Chronic HBV infection can range from asymptomatic carrier state to continuous hepatocellular necrosis and inflammation, and in some instances may lead to hepatocellular carcinoma. Children exposed to HBV infection, particularly those less than one year old, often develop chronic infection and represent the major source of chronic infection. Worldwide, nearly 200 million people are chronically infected with HBV. And finally, in a small percentage of HBV infections (0.1 -0.5%) a fulminant hepatitis results in such extreme cell death in the liver that fewer than one-fifth to one-third of these patients survive.

specificity of the cytolytic activity, its HLA restriction elements, and cellular phenotype were not established. See, Mondelli et al., J. Immunol. 129:2773 (1982) and Mondelli et al., Clin. Exp. Immunol. 6:311 (1987). More recently, Moriyama et al., Science 248:361-364 (1990), reported that the HBV major envelope antigen was expressed at the hepatocyte surface in a form recognizable by MHC class I-restricted, $CD8^+$ cytotoxic T lymphocytes, and by envelope-specific antibodies. However, the HBV epitopic regions responsible for HBV-specific CTL activity were not identified.

The requirement for lymphokines such as IL-2 in the generation of CD8+ CTL is well established, although the need for activation of CD4+ T helper cells to provide these lymphokines remains somewhat controversial. While the concept of linked T helper-B cell recognition for antibody production has been firmly defined, there is no compelling evidence for linked T helper-CTL recognition for the in vivo induction of CD8+ CTL. See, e.g., Buller et al., Nature 328:77-79 (1987); Sarobe et al., Eur. J. Immunol. 21:1555-1558 (1991); and Cassell and Forman, Annals N.Y. Acad. Sci. :51-60 (1991).

Individuals chronically infected with HBV are at risk of developing liver cirrhosis and/or hepatocellular carcinoma, and constitute an extremely large reservoir for spreading the disease. It would be desirable to stimulate the immune systems of those chronically infected to respond to appropriate HBV antigens and eliminate their infections, or to be able to prevent the evolution from an acute HBV infection to the chronic stage. Further, as the presently approved HBV vaccines provide only about 90% protection among those immunized, it is desirable to improve the existing vaccines by increasing or diversifying the immunogenicity of the vaccines to elicit a more effective immunity. Means are also needed for predicting which patients with acute HBV infection are likely to develop chronic HBV infection, so that appropriate treatment and precautions can be implemented earlier. Quite

heteropolymers. In some instances peptides will be combined in a composition as an admixture and will not be linked. The CTL inducing peptide can also be linked to a lipid-containing molecule capable of enhancing a T lymphocyte response, or may be linked to a T helper peptide which induces a T-helper cell response, or may be linked to both a lipid-containing molecule and a T helper peptide, for example. Linkage to a lipid or a T-helper peptide may be either at the amino or carboxy termini.

Compositions are provided which comprise a peptide of the invention formulated with an additional peptide, a liposome, an adjuvant and/or a pharmaceutically acceptable carrier. Thus, pharmaceutical compositions can be used in methods of treating acute HBV infection, particularly in an effort to prevent the infection from progressing to a chronic or carrier state. Methods for treating chronic HBV infection and HBV carrier states are also provided, where the pharmaceutical compositions are administered to infected individuals in amounts sufficient to stimulate immunogenically effective CTL responses against HBs and HBc epitopes. For treating these infections it may be particularly desirable to combine the peptides which induce MHC class I restricted CTL responses against HBV antigen with other peptides or proteins that induce immune response to other HBV antigens. To treat individuals with chronic or carrier state infections the compositions may be administered with an initial dosage followed by a boosting dosage over a period of time, as necessary to resolve or substantially mitigate the infection.

Vaccine compositions for preventing HBV infection, including preventing development of chronic HBV infection from an acute infection, are also provided. The vaccine compositions comprise an immunogenically effective amount of a HBV peptide which induces a MHC class I restricted CTL response. In the case of HLA-A2 haplotype individuals, the peptide can be derived from any of peptides 799.08 ($HBenv_{309-328}$) [Seq. ID No. 1], 799.09 ($HBenv_{329-348}$) [Seq. ID No. 7], 799.10 ($HBenv_{349-368}$) [Seq. ID No. 2], and/or 802.03

Brief Description of the Drawings

Fig. 1 depicts the results of induction of HBV peptide-specific A2.1-restricted CTL by priming A2.1/K^b transgenic mice with syngeneic spleen cells "loaded" with HBV. Panels A-D: Splenocytes from HBV-primed transgenic mice were restimulated in vitro with four mixtures of syngeneic LPS blasts each coated with one of 13 different peptides. After 9 days effector cells were assayed for lytic activity against ^{51}Cr labelled Jurkat A2.1/K^b target cells in the presence or absence of the four different peptide mixtures used for induction. Panels E-M: Effector cells raised against the four different peptide mixtures were restimulated in vitro against the same peptide mixtures and assayed for lytic activity against ^{51}Cr labelled Jurkat A2.1/K^b target cells in the presence or absence of the individual peptides.

Fig. 2 illustrates the HBV peptide specificity of A2.1 transgenic CTL. Transgenic CTL raised from HBV-primed transgenic mice and restimulated in vitro twice with one of the four different peptide mixtures were restimulated with individual HBV peptides and assayed for lytic activity on ^{51}CR labelled Jurkat target cells in the presence or absence of the HBV peptides used for the restimulation.

Fig. 3 illustrates the results of induction of HBV peptide-specific A2.1-restricted CTL by priming A2.1/K^b transgenic mice with HBV in IFA. A. Splenocytes from HBV-primed transgenic mice were restimulated in vitro with syngeneic LPS blasts coated with HBV peptides. After 6d, effector cells were assayed for lytic activity against ^{51}Cr labelled Jurkat A2.1/K^b target cells in the presence or absence of the appropriate HBV peptide. Each panel represents the CTL activity induced by the indicated target peptide.

Fig 4. The effector CTL of Fig. 3 were restimulated with peptide coated LPS blasts followed at a one week interval by restimulation with peptide coated Jurkat A2.1/K^b cells. Six days after the last restimulation, effector cells were

were linked via peptide bonds using an exemplary spacer such as alanine-alanine-alanine. Protocol was similar to that in Figs. 5 and 6.

Fig. 10 illustrates that previous priming of helper T cells was not required for in vivo priming of HBc 18-27-specific CTL responses using peptide 902.01 and 902.02. CTL response is shown from animals primed subcutaneously with peptide 902.01 (Fig. 10A) or 902.02 (Fig. 10B) alone without the previous priming with peptide 875.23 in CFA.

Fig. 11 illustrates the induction of $HBenv_{360-368}$ specific CTL response. A2.K^b transgenic mice were injected with 100 microliters of an emulsion (IFA) of 100 mg HBenv360-368 and 100 mg HBc128-140. Three weeks later, splenocytes were restimulated with syngeneic LPS blasts coated with peptide HBenv360-368. Effector cells were assayed for cytotoxicity against ^{51}Cr labeled Jurkat A2/K^b target cells in the presence or absence of HBenv 360-368.

Fig. 12 illustrates the induction of a CTL response specific for HBc 18-27 by priming with a peptide containing HBc 18-27 linked to tetanus toxoid 830-843 (human helper T cell epitope). Effector cells wee assayed against 51Cr labeled Jurkat A2-1/Kb target cells in the present or absence of HBc 18-27; Jy target cells in the presence or absence of HBc 18-27 and Jy cells that had been transfected with HBV core.

Fig. 13 illustrates the minimal sequence for CTL recognition within HBV env 329-348 peptide (799.09). CTL lines 110 and 113 were derived from splenocytes obtained from A2Kb transgenic mice primed subcutaneously with HBV virus in IFA and in vitro activated with 799.09 coated stimulator cells. 799.09 specific CTL lines 110 and 113 were assayed for lytic activity in a 6 hr 51Cr release assay using JA2Kb cells as targets in the presence of 799.09 peptide truncations (Panel A = 799.09 N-terminus truncations; Panel B = 799.09 overlapping 9 mers and 10 mers).

carcinoma, breast cancer, carcinoembryonic antigens, melanoma (MAGE-1) antigens, and prostate cancer specific antigen, hepatitis C antigens, Epstein-Barr virus antigens, HIV-1 and HIV-2 antigens, and papilloma virus antigens.

With respect to hepatitis B, as set forth in more detail below, usually at least four, sometimes six, often seven or more residues of the peptide or a majority of amino acids of that peptide will be identical or homologous when compared to the corresponding portion of the naturally occurring HBenv sequence identified as $HBenv_{309-328}$ (peptide 799.08) or $HBenv_{329-349}$ (peptide 799.09) or $HBenv_{349-368}$ (peptide 799.10), or the HBc region HBc_{91-110} (peptide 802.03).

The peptides can be prepared "synthetically," as described hereinbelow, or by recombinant DNA technology. Although the peptide will preferably be substantially free of other naturally occurring HBV proteins and fragments thereof, in some embodiments the peptides can be synthetically conjugated to native fragments or particles. The term peptide is used interchangeably with polypeptide in the present specification to designate a series of amino acids connected one to the other by peptide bonds between the alpha-amino and alpha-carboxy groups of adjacent amino acids. The polypeptides or peptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the polypeptides as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the biological activity of the large peptide. When possible, it may be desirable to optimize peptides of the invention to a length of eight to twelve amino acid residues, commensurate in size with endogenously processed viral peptides that are bound to MHC class I molecules on the cell surface. See generally,

799.08 ($HBenv_{309-328}$) [Seq. ID No. 1]
Asn-Cys-Thr-Cys-Ile-Pro-Ile-Pro-Ser-Ser-Trp-Ala-
Phe-Gly-Lys-Phe-Leu-Trp-Glu-Trp

wherein the peptide can be optionally flanked and/or modified at one or both of the N- and C-termini, as desired, by amino acids from HBV sequences, particularly HBenv, amino acids added to facilitate linking, other N- and C-terminal modifications, linked to carriers, etc., as further described herein. For a peptide of the HBV subtype adw, Gly_{322} is replaced by Ala, and Phe_{324} is replaced by Tyr. The peptide $HBenv_{309-328}$ induces a CTL response which is mediated by at least the MHC class I molecule HLA-A2.

Another HBenv CTL inducing peptide embodiment of the invention comprises from six to twenty amino acids of the 799.10 peptide region $HBenv_{349-368}$, and includes peptides derived from $HBenv_{349-368}$ which contain an epitopic site(s) of at least seven or more amino acids where a majority of amino acids of the peptide will be identical or homologous when compared to the corresponding portion of the naturally occurring HBenv sequence identified as $HBenv_{349-368}$, which is as follows (for HBV subtypes ayw and adw):

799.10 ($HBenv_{349-368}$) [Seq. ID No. 2]
Leu-Ser-Pro-Thr-Val-Trp-Leu-Ser-Val-Ile-
Trp-Met-Met-Trp-Tyr-Trp-Gly-Pro-Ser-Leu

wherein the peptide selected from said region can be flanked and/or modified at one or both termini as described herein. An example of another CTL inducing peptide derived from the region of 799.10 ($HBenv_{349-368}$) [Seq. ID No. 2] which contains at least one epitope capable of inducing a MHC class I-restricted cytotoxic T-lymphocyte response to hepatitis B virus is:

884.02 ($HBenv_{349-358}$) [Seq. ID No. 3]
Leu-Ser-Pro-Thr-Val-Trp-Leu-Ser-Val-Ile.

883.02 (HBc_{92-101}) [Seq. ID No. 5]
Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu,

(HBc_{92-100}) [Seq. ID No. 9]
Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu, and

883.03 (HBc_{93-102}) [Seq. ID No. 6]
Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu-Trp.

As mentioned above, additional amino acids can be added to the termini of an oligopeptide or peptide to provide for ease of linking peptides one to another, for coupling to a carrier, support or larger peptide, for reasons discussed herein, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal-NH_2 acylation, e.g., by alkanoyl (C_1-C_{20}) or thioglycolyl acetylation, terminal-carboxy amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

It will be understood that the peptides of the present invention or analogs thereof which have CTL stimulating activity may be modified to provide other desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide. For instance, the peptides can be modified by extending, decreasing or substituting in the peptides amino acid sequences by, e.g., the addition or deletion of amino acids on either the amino terminal or carboxy terminal end, or both, of peptides derived from the sequences disclosed herein. As further described below, the CTL activity of the subject peptides can be enhanced by linkage to a sequence which contains at least one

region 799.08 ($HBenv_{309-328}$), peptide region 799.09 ($HBenv_{329-348}$) peptide region 799.10 ($HBenv_{349-368}$), or peptide region 802.03 (HBc_{91-110}), there are residues (or those which are substantially functionally equivalent) which allow the peptide to retain their biological activity, i.e., the ability to stimulate a class I-restricted cytotoxic T-lymphocytic response against HBV infected cells or cells which express HBV surface and/or nucleocapsid antigens. These residues can be identified by single amino acid substitutions, deletions, or insertions. In addition, the contributions made by the side chains of the residues can be probed via a systematic scan with a specified amino acid (e.g., Ala). Peptides which tolerate multiple substitutions generally incorporate such substitutions as small, relatively neutral molecules, e.g., Ala, Gly, Pro, or similar residues. The number and types of residues which can be substituted, added or subtracted will depend on the spacing necessary between the essential epitopic points and certain conformational and functional attributes which are sought (e.g., hydrophobicity vs. hydrophilicity). If desired, increased binding affinity of peptide analogues to its MHC molecule for presentation to a CTL can also be achieved by such alterations. Generally, any spacer substitutions, additions or deletions between epitopic and/or conformationally important residues should employ amino acids or other moieties chosen to avoid steric and charge interference which might disrupt binding.

Peptides which tolerate substitutions while retaining the desired biological activity may also be synthesized as D-amino acid containing peptides. Such peptide may be synthesized as "inverso" or "retro-inverso" forms, that is, by replacing L-amino acids of a sequence with D-amino acids, or by reversing the sequence of the amino acids and replacing the L-amino acids with D-amino acids. As the D-peptides are substantially more resistant to peptidases, and therefore are more stable in serum and tissues compared to their L-peptide counterparts, the stability of D-peptides under physiological conditions may more than compensate for a difference in

Linkages for homo- or hetero-polymers or for coupling to carriers can be provided in a variety of ways. For example, cysteine residues can be added at both the amino- and carboxy-termini, where the peptides are covalently bonded via controlled oxidation of the cysteine residues. Also useful are a large number of heterobifunctional agents which generate a disulfide link at one functional group end and a peptide link at the other, including N-succidimidyl-3-(2-pyridyldithio) proprionate (SPDP). This reagent creates a disulfide linkage between itself and a cysteine residue in one protein and an amide linkage through the amino on a lysine or other free amino group in the other. A variety of such disulfide/amide forming agents are known. See, for example, Immun. Rev. 62:185 (1982). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thioether forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2 bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimido-methyl) cyclohexane- 1-carboxylic acid and the like. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxy-2-nitro-4-sulfonic acid, sodium salt. A particularly preferred coupling agent is succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). Of course, it will be understood that linkage should not substantially interfere with either of the linked groups to function as described, e.g., to function as an HBV cytotoxic T cell determinant, peptide analog CTL antagonist, or HBV T helper determinant.

In another aspect the peptides of the invention can be combined or coupled with other peptides which present HBV T-helper cell epitopes, i.e., T helper peptides comprising six to thirty amino acids containing a T helper epitope which from the envelope, core or other immunogenic protein or derivative thereof, stimulate T cells that cooperate in the induction of cytotoxic T cells to HBV. The T-helper cells can be either the T-helper 1 or T-helper 2 phenotype, for example.

mimetics, which are substantially uncharged under physiological conditions and may have linear or branched side chains. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. In certain preferred embodiments herein the neutral spacer is Ala. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. Preferred exemplary spacers are homo-oligomers of Ala. When present, the spacer will usually be at least one or two residues, more usually three to six residues. In other embodiments the T helper peptide is conjugated to the CTL peptide, preferably with the T helper peptide positioned at the amino terminus. The peptides may be joined by a neutral linker, such as Ala-Ala-Ala or the like, and preferably further contains a lipid residue such as palmitic acid or the like (as described further below) which is attached to alpha and epsilon amino groups of a Lys residue ($(PAM)_2Lys$), which is attached to the amino terminus of the peptide conjugate, typically via Ser-Ser linkage or the like.

The CTL inducing peptide may be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the CTL inducing peptide or the T helper peptide may acylated. In addition, the CTL peptide/T helper conjugate may be linked to certain alkanoyl (C_1-C_{20}) lipids via one or more linking residues such as Gly, Gly-Gly, Ser, Ser-Ser as described below.

In an exemplary embodiment described below, a T helper peptide from substantially within $HBc_{128-140}$ (Thr-Pro-Pro-Ala-Tyr-Arg-Pro-Pro-Asn-Ala-Pro-Ile-Leu) [Seq. ID No. 19]), when linked with a CTL peptide (HBc18-27), was shown to induce specific CTL priming of animals in all animals studied, and at levels which were greater than when the CTL peptide and T helper peptide were administered unlinked. When the T helper and CTL HBV peptides were linked by a Ala-Ala-Ala spacer, specific CTL activity greater than induction of specific CTL

response. By this means a polypeptide is used which incorporates several T cell epitopes.

As the coding sequence for peptides of the length contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

The peptides of the present invention and pharmaceutical and vaccine compositions thereof are useful for administration to mammals, particularly humans, to treat and/or prevent HBV infection. As the peptides are used to stimulate cytotoxic T-lymphocyte responses to HBV infected cells, the compositions can be used to treat or prevent acute and/or chronic HBV infection.

For pharmaceutical compositions, the peptides, i.e., the CTL peptide or CTL/T helper peptide, of the invention as described above will be administered to an individual already infected with HBV. Those in the incubation phase or the acute phase of infection can be treated with the immunogenic peptides separately or in conjunction with other treatments,

of an effective CTL response to HBV during treatment of acute hepatitis will minimize the possibility of subsequent development of chronic hepatitis, HBV carrier stage, and ensuing hepatocellular carcinoma.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, for instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

The peptide compositions can also be used for the treatment of chronic hepatitis and to stimulate the immune system of carriers to eliminate virus-infected cells. Those with chronic hepatitis can be identified as testing positive for virus from about 3-6 months after infection. As individuals may develop chronic HBV infection because of an inadequate (or absent) CTL response during the acute phase of their infection, it is important to provide an amount of immuno-potentiating peptide in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response. Thus, for treatment of chronic hepatitis, a representative dose is in the range of about 1.0 μg to about 500 μg, preferably about 5 μg to 100 μg for a 70 kg patient per dose. Administration should continue until at least clinical symptoms or laboratory indicators indicate that the HBV infection has been eliminated or substantially abated and for a period thereafter. Immunizing doses followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a prolonged period of time, as necessary to resolve the infection. For the treatment of chronic and carrier HBV infection it may also be desirable to combine the CTL peptides with other peptides or

are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the CTL stimulatory peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

In some embodiments it may be desirable to include in the pharmaceutical composition at least one component which primes CTL. Lipids have been identified as agents capable of priming CTL in vivo against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., typically via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to a synthetic peptide which comprises a class I-restricted CTL epitope. As further described herein, the lipidated peptide can then be incorporated into a liposome emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of a class I restricted peptide having T cell determinants, such as those peptides described herein as well as other peptides which have been identified has having such determinants.

selectively inserted between the lipid moiety and the CTL or T helper peptide, as well as between the T helper and the CTL inducing peptides. In the case of the spacer between the lipid and the T helper or CTL inducing peptide, a preferred example comprises Lys-Ser-Ser, although other spacers are described herein. An example of a spacer between the T helper and CTL inducing peptides will be Ala-Ala-Ala, as also described in further detail herein. The CTL inducing peptide can be from the HBc or HBs region, or from other CTL inducing antigens as noted above.

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 1%, usually at or at least about 10% to as much as 20 to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 100 mg of peptide. Actual methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, PA (1985), which is incorporated herein by reference.

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, such as lymphoid tissue, or targeted selectively to HBV infected hepatic cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic

surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of a CTL stimulating peptide as described herein. The peptide(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies and/or cytotoxic T cells that react with different antigenic determinants of HBV. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(D-lysine:D-glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. And, as mentioned above, CTL responses can be primed by conjugating peptides of the invention to lipids, such as P_3CSS. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing large amounts

sequences that encode the peptides or conjugates of the invention. Upon introduction into an acutely or chronically HBV-infected host or into a non-infected host, the recombinant vaccinia virus expresses the HBs and/or HBc peptide, and thereby elicits a host CTL response to HBV. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (bacille Calmette Guerin). BCG vectors are described in Stover et al. (Nature 351:456-460 (1991)) which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., Salmonella typhi vectors and the like, will be apparent to those skilled in the art from the description herein.

The peptides may also find use as diagnostic reagents. For example, a peptide of the invention may be used to determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In addition, the peptides may also be used to predict which individuals will be at substantial risk for developing chronic HBV infection.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE I

Identification of CTL-Specific HBV Epitopes

A line of transgenic mice which express a mouse-human chimeric class I molecule was used to define HBV core and surface antigen sequences that represent CTL-specific epitopes.

The transgenic mouse line 66 obtained from Scripps Clinic and Research Foundation expresses a chimeric class I molecule composed of the α1 and α2 domains of human HLA-A2.1 antigen and the α3 transmembrane and cytoplasmic domains of H-

0.2M TrisHCl pH 8.1, 2.0×10^{-4} N-benzyloxycarbonyl-L-Lysine thiobenzyl ester (BLT) and 2.2×10^{-4} M dithiobis (nitrobenzoic acid). Plates were incubated for 1 hour at 37C and absorbance read at 412 nm. Percent inhibition was calculated by the following formula:

$$\% \text{ inhibition} = 100 - \frac{A_{412} \text{ (test + index) peptide} - A_{412} \text{ test peptide alone}}{A_{412} \text{ index peptide} - A_{412} \text{ no peptide}} \times 100$$

Those peptides which bound to A2.1 and caused more than 24% inhibition of serine esterase release by the cells were assayed in vitro for the ability to restimulate a CTL response from splenocytes derived from HBV primed A2.1 transgenic mice. (Sette, A. et al., J. Immunol. 147:3893 (1991)). HBV priming was performed by injecting A2.1 spleen cells "loaded" with HBV virus as described by Carbone and Bevan, J. Exp. Med. 171:377-387 (1990).

Briefly, red blood cell depleted splenocytes were suspended in .4 ml of a solution composed of 200 µl of HBV purified virus and 200 µl of a 2 x hypertonic solution (0.5 M sucrose, 10% w/v polyethylene glycol 1000, 10 mM Hepes, pH 7.2, in RPMI 1640 medium), for 10 min. at 37C. The cell suspension was then rapidly diluted in prewarmed hypotonic media (60% HBSS and 40% water), incubated for 2 min. at 37C, pelleted, washed twice in HBSS and irradiated (1,000 rad.). Mice were then injected with 5.0×10^6 loaded cells in a volume of 200 µl. Mice were boosted with HBV-loaded spleen cells 10 days later.

After about 2 weeks, spleen cells from primed mice (5×10^6 cells/well in 24 well plates) were cultured with 4 different mixtures of syngeneic irradiated (3000 rads) LPS blasts (2×10^6 cells/well) that had been independently coated with 13 different peptides. Coating was achieved by incubating aliquots of 25×10^6 LPS blasts in tubes each with 100 µg of one of the 13 HBV synthetic peptides in one mL for

(panel F), HBenv 329-348 and 349-368 (panel G) and HBenv 309-328 (panel H) were independently used to restimulate the effector cells generated with the peptide mixtures. After 6d in culture, the effector cells were tested for cytotoxicity against ^{51}Cr Jurkat A_2/K^b cells in the presence of the peptide used for the restimulation (Fig. 1). The set of experiments, outlined in this example allow us to determine that HBV peptides HBc 11-27 (Fig. 1 panels A,E; Fig. 2 panel J) HBc 91-110 (Fig. 1 panels B,F; Fig. 2 panel M), HBenv 329-348 (Fig. 1 panels C,G; Fig. 2 panel N) HBenv 349-368 (Fig. 1 panels C,G; Fig. 2 panel O) and HBenv 309-328 (Fig. 1 panels D,H; Fig. 2 panel P) clearly represent CTL epitopes.

EXAMPLE II

Induction of A2.1-restricted CTL by Subcutaneous Priming with Purified HBV in Incomplete Freund's Adjuvant (IFA)

Injection of ovalbumin (OVA) in IFA subcutaneously induces an ovalbumin-specific CTL response in mice, while injection of OVA either i.v. or i.p. generally does not lead to the generation of CTL. This technique was used to induce HBV-specific CTL in A2.1 transgenic mice.

Priming and In Vitro Restimulation: $A2.1/K^b$ transgenic mice were injected with 100 microliters of an emulsion of purified HBV virus in incomplete Freund's adjuvant (IFA). This emulsion was prepared by mixing purified HBV (1 mg protein/ml) diluted 1:5 in HBSS with an equal volume of IFA. Seven days after priming, splenocytes ($5x10^6$ cells/well in a 24 well plate) obtained from these animals were restimulated with syngeneic irradiated LPS blasts ($2x10^6$/well) coated with each of the following peptides:

799.09	HBenv 329-348	802.03	HBc 91-110
875.20	HBenv 335-343	883.02	HBc 92-101
875.21	HBenv 338-347	883.03	HBc 93-102
799.10	HBenv 349-368	875.15	HBc 18-27
884.01	HBenv 348-357	875.18	HBc 107-115
884.02	HBenv 349-358	875.19	HBc 139-148

EXAMPLE III

Synthesis of Peptides

Peptides were synthesized on an Applied Biosystems (Foster City, CA) 430A peptides synthesizer using Fmoc protected amino acids and 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) esters for amino acid activation. Each amino acid was routinely triple coupled. Fmoc protected amino acids and Hydroxybenzotriazole were purchased from Burdick and Jackson. HBTU was purchased from Richelieu Biotechnologies (St-Hyacinthe, Canada). Piperidine and trifluoroacetic acid, acetic anhydride, and ethanedithiol were purchased from Sigma Chemical Corporation.

a. Peptide Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val-OH [Seq. ID No. 23]

L-Valine coupled to Sasrin® resin (Bachem Biosciences) was loaded into the peptide synthesis reaction vessel and washed one time with N-methylpyrolidone (NMP). The following operations were then sequentially performed:

1. The Fmoc protecting group was removed by treatment of the resin bound amino acid with 25% piperidine in NMP.
2. The resin was washed 5 times with NMP.
3. A mixture containing Fmoc-serine, diisopropylethylamine, HBTU and NMP was added to the reaction vessel and allowed to react for 30 minutes, under vortex agitation.
4. The solvent was drained, and the resin was washed three times with NMP.
5. Steps (3) and (4) were repeated two more times.
6. The resin was washed four more times with NMP.

Steps 1-6 were repeated for each amino acid of the peptide. Following the final coupling cycle, the resin-bound peptide was deproteced by reaction with 25% piperidine in NMP, washed 7 times with NMP, and washed 2 times with dichloromethane. The resin was dried in vacuo for 24 hours. The peptide was

The resin bound peptide described in section a was chain extended by the addition of Glu, Thr, Ile, Gly, Ile, Phe, Lys, Ser, Asn, Ala, Lys, Ile, Tyr, and Gln residues, according to the procedure described in Section a. Cleavage and purification were performed as described above.

d. Peptide Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Ala-Ala-Ala-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val-OH [Seq. ID No. 26].

The resin bound peptide described in section a was chain extended by the sequential addition of Ala, Ala, Ala, Glu, Thr, Ile, Gly, Ile, Phe, Lys, Ser, Asn, Ala, Lys, Ile, Tyr, and Gln residues, according to the procedure described in section a. Cleavage and purification were performed as described above.

e. Peptide Ac-Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Ala-Ala-Ala-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val-OH [Seq. ID No. 27].

The resin bound peptide described in Section d was acetylated by reaction with acetic anhydride in NMP. Cleavage and purification were performed as described above.

EXAMPLE IV

Induction of CTL By Combining CTL and T-Helper Epitopes

This example describes experiments which define the relative in vivo HBV-specific CTL priming efficiency of peptides expressing HBV CTL epitopes alone, CTL epitopes mixed with peptides containing T helper epitopes or CTL epitopes physically linked to T helper epitopes.

Transgenic mice (HLA-A2.1/K^b) were primed subcutaneously (base of tail) with 100 μg of peptide 875.23 (Ia^b-restricted helper epitope HBc 128-140 TPPAYRPPNAPIL) in

complete Freund's adjuvant (CFA). Nine days later each of the following peptides were injected subcutaneously into two unprimed and two helper-primed mice, 100 μg/mouse in incomplete Freund's adjuvant (IFA).

Peptide	T Helper (HBc 128-140)		CTL (HBc 18-27)
1. 875.23	TPPAYRPPNAPIL		
2. 875.15			FLPSDFFPSV
3. 875.23+875.15	TPPAYRPPNAPIL	+	FLPSDFFPSV
4. 902.01 =	TPPAYRPPNAPILFLPSDFFPSV-NH_2		
5. 902.02	TPPAYRPPNAPILAAAFLPSDFFPSV-NH_2		
6. No peptide			

Three weeks after priming with the CTL epitope, splenocytes were in vitro restimulated with LPS blasts coated with HBc 18-27 (coating was achieved by incubating 30 x 10^6 LPS blasts with 100 μg of HBc18-27 in one ml of medium; after 1-2 hr at 37C, the cells were washed). After 6 days, effector cells were assayed for lytic activity against ^{51}Cr labelled Jurkat A_2/K^b target cells in the presence or absence of HBc18-27.

The results showed that in 50% of the animals studied in which the T helper and CTL epitope peptides were simply mixed (i.e., not linked) and administered in an immunizing dose, induction of some detectible antigen-specific CTL activity above the level of background killing was seen. An example of the response detected is shown in Fig. 7. Surprisingly, when animals were primed with the T helper epitope linked to the CTL epitope, 100% showed evidence of specific CTL priming (Fig. 8), the magnitude of which was greater than that detected when the epitopes were administered non-linked (Fig. 7). Quite unexpectedly, as shown in Fig. 9, it was found that linking the T helper and CTL epitopes via an alanine-alanine-alanine spacer (i.e., T helper-AAA-CTL) resulted in the induction of specific CTL activity greater than that detected by linking the T helper and CTL determinants alone. Priming with the T helper peptide or CTL

Three weeks after priming, splenocytes were restimulated in vitro with LPS blasts coated with HBc 18-27 (as described in example I). After 7 days cells were restimulated with jurkat A2/Kb cells coated with HBc 18-27 (as described in Example I) - After 6 days these effector cells were assayed for cytotoxicity against 51Cr labeled jurkat A2/Kb target cells in the presence or absence of HBc 18-27, Jy target cells in the presence or absence of HBc 18-27 and Jy cells transfected with HBV core. The results, shown in Fig. 12, indicate that peptide 934.02 effectively induces CTL specific for HBc 18-27. Moreover, these CTL recognize and kill endogenously presented antigen (Jy core).

Example VII

Comparison of CTL Immunity Induced by Peptide Immunization

Various modifications and formulations of an antigenic CTL peptide were tested in an effort to enhance its immunogenicity. BALB/c mice were primed subcutaneously in the base of the tail with one of the following peptides or peptide mixtures:

Table I

CTL Immunogenicity of Various Modifications and Formulations of PR8-NP 148-155

Peptide	Formulation					
	Saline		Alum		IFA	
	0.01[a]	0.1	0.01	0.1	0.01	0.1
932.01	-[b]	-	-	-	+	+++
932.07	++	+	-	-	-	-
932.01 + 577.01	-	++	-	-	+++	++
932.07 + 577.01	+	-	-	-	-	±
932.02	-	-	-	+++	+++	+++
932.04	+++	+++	+++	+++	+++	+++
932.03	-	-	-	±	+++	++
932.05	+++	+++	+++	+++	+++	+++

a = Dose (μM/mouse)

b = CTL immunogenicity of various modifications and formulations of NP 148-155 (nucleoprotein of PR8 influenza virus). Each symbol represents the result obtained from spleen cells derived from a single Balb/c mouse and reflects the effector to target ratio (E:T) required to induce 40% antigen specific lysis of ^{51}Cr labeled B10D2 target cells in the presence of $ND_{148-155}$ peptide; +++ E:T below 10:1; ++ E+T between 10+1 and 30:1; + E:T greater than 30:1; - not achieved at any E:T tested.

The constructs (PAM_2KSS-T helper-CTL and $(PAM)_2$KSS-T helper-AAA-CTL were superior when injected in saline or Alum compared to all of the other combinations. The peptides T helper-CTL and T helper-AAA-CTL were superior to mixing the T helper + CTL (i.e., non-linked) and worked well in IFA, but not well in saline or Alum. Thus, for vaccine development, linking the $(PAM)_2$KSS to a T helper peptide which is linked to the CTL peptide appears to be advantageous for inducing CTL immunity.

SEQUENCE LISTING

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Chesnut, Robert W.

(ii) TITLE OF INVENTION: HLA-RESTRICTED HEPATITIS B VIRUS CTL EPITOPES

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE: 26-AUG-1992
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/749,568
(B) FILING DATE: 26-AUG-1991

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/827,682
(B) FILING DATE: 29-JAN-1992

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/874,491
(B) FILING DATE: 27-APR-1992

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Gly Lys Phe

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn Met Gly Leu Lys Phe Arg Gln Leu Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Gly Leu Lys Phe Arg Gln Leu Leu Trp
1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Ser Ala Arg Phe Ser Trp Leu Ser Leu Leu Val Pro Phe Val Gln
1 5 10 15

Trp Phe Val Gly
20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Trp Leu Ser Leu Leu Val Pro Phe Val
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala Tyr Arg Pro Pro
1 5 10 15

Asn Ala Pro Ile
20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Thr Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Phe Leu Pro
1 5 10 15

Ser Asp Phe Phe Pro Ser Val
20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Thr Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ala Ala Ala
1 5 10 15

Phe Leu Pro Ser Asp Phe Phe Pro Ser Val
20 25

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Trp Met Met Trp Tyr Trp Gly Pro Ser Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Phe Leu Pro Ser Asp Phe Phe Pro Ser Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Ala Ala
1 5 10 15

Ala Phe Leu Pro Ser Asp Phe Phe Pro Ser Val
20 25

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Lys Ser Ser Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr
1 5 10 15

Glu Ala Ala Ala Phe Leu Pro Ser Asp Phe Phe Pro Ser Val
20 25 30

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Ile Ser Gln Ala Val His Ala Ala His Ala Glu Ile Asn Glu
1 5 10

7. The peptide of claim 5, which is
923.22 ($HBenv_{335-343}$) [Seq. ID No. 8]
Trp-Leu-Ser-Leu-Leu-Val-Pro-Phe-Val.

8. The peptide of claim 5, which is
923.27 ($HBenv_{332-341}$) [Seq. ID No. 33]
Arg-Phe-Ser-Trp-Leu-Ser-Leu-Leu-Val-Pro.

9. A CTL/T helper peptide conjugate comprising a CTL inducing peptide linked to a T helper peptide.

10. The CTL/T helper peptide conjugate of claim 9 wherein the CTL inducing peptide is 799.09 ($HBenv_{323-348}$).

11. The CTL/T helper peptide conjugate of claim 9 wherein the CTL inducing peptide is 799.10 ($HBenv_{349-368}$).

12. The CTL/T helper peptide conjugate of claim 9 wherein the CTL inducing peptide is 923.27 ($HBenv_{332-341}$).

13. The CTL/T helper peptide conjugate of claim 9 wherein the CTL inducing peptide is 917.07 ($HBenv_{360-368}$).

14. The CTL/T helper peptide conjugate of claim 9 wherein the CTL inducing peptide is 875.15 (HBc_{18-27}).

15. The CTL/T helper peptide conjugate of claim 9 wherein the T helper peptide is hepatitis B core peptide selected from the group consisting of (HBc_{1-20}), (HBc_{50-69}) or ($HB_{c111-125}$).

16. The CTL/T helper peptide conjugate of claim 9 wherein the T helper peptide is tetanus toxoid ($TT_{830-843}$).

17. The CTL/T helper peptide conjugate of claim 14, wherein the T helper peptide is tetanus toxoid ($TT_{830-843}$).

27. The CTL/T helper peptide conjugate of claim 9, wherein the CTL inducing peptide is 799.08 ($HBenv_{309-328}$).

28. A CTL inducing peptide comprising from six to twenty amino acids wherein at least a majority of the amino acids of said CTL inducing peptide are homologous to the corresponding portion of 802.03 (HBc_{91-110}) having the following sequence:

802.03 (HBc_{91-110}) [Seq. ID No. 4]
Thr-Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu-Trp-Phe-His-Ile-Ser-Cys-Leu-Thr-Phe.

29. The peptide of claim 28, which is
802.03 (HBc_{91-110}) [Seq. ID No. 4]
Thr-Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu-Trp-Phe-His-Ile-Ser-Cys-Leu-Thr-Phe.

30. The peptide of claim 28, which is
883.02 (HBc_{92-101}) [Seq. ID No. 5]
Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu.

31. The peptide of claim 28, which is
(HBc_{92-100}) [Seq. ID No. 5]
Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu.

32. The peptide of claim 28, which is
883.03 (HBc_{93-102}) [Seq. ID No. 6]
Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu-Trp.

33. A pharmaceutical composition for the treatment of hepatitis B virus infection, which comprises a CTL inducing peptide according to claims 1, 5, 25 or 28 and a pharmaceutically acceptable carrier.

34. A pharmaceutical composition for the treatment of hepatitis B virus infection comprising a CTL/T helper peptide conjugate and a physiologically acceptable carrier.

46. The pharmaceutical composition of claim 36, wherein the T helper peptide is from tetanus toxoid.

47. The pharmaceutical composition of claim 33, wherein the carrier is a liposome.

48. The pharmaceutical composition of claim 33, wherein the peptide is conjugated to the carrier.

49. The pharmaceutical composition of claim 48, wherein the carrier is a lipid.

50. The pharmaceutical composition of claim 49, wherein the lipid is comprised of palmitic acid attached to epsilon and alpha amino groups of a Lys residue, wherein the Lys is linked to the amino terminus of the peptide by means of a Ser-Ser linker.

51. A method of treating hepatitis B infection, comprising administering an effective amount of a CTL inducing peptide according to claims 1, 5, 25 or 28 to an HBV infected host.

52. The method of claim 51, wherein the infected host has chronic hepatitis B infection.

53. The method of claim 51, wherein the infected host has acute hepatitis B infection.

54. The method of claim 51, wherein the CTL inducing peptide is administered prophylactically.

55. A method of treating hepatitis B infection, comprising administering an effective amount of a CTL/T helper peptide conjugate.

63. The CTL/T helper/lipid conjugate of claim 61, wherein the spacer molecule is Lys-Ser-Ser.

64. The CTL/T helper/lipid conjugate of claim 61, wherein the CTL inducing peptide is linked to the T helper peptide by a spacer molecule.

65. The CTL/T helper/lipid conjugate of claim 64, wherein the spacer molecule between the CTL inducing peptide and the T helper peptide is Ala-Ala-Ala.

66. The CTL/T helper/lipid conjugate of claim 60, wherein CTL inducing peptide is linked to the T helper peptide by a spacer molecule.

67. The CTL/T helper/lipid conjugate of claim 66, wherein the spacer molecule between the CTL inducing peptide and the T helper peptide is Ala-Ala-Ala.

68. The CTL/T helper/lipid conjugate of claim 60, wherein CTL inducing peptide is HBc18-27.

69. A pharmaceutical composition for the treatment of hepatitis B infection which comprises a CTL/T helper/lipid conjugate comprising a linked CTL inducing peptide, T helper peptide and a lipid, and a physiologically acceptable carrier.

70. The pharmaceutical composition of claim 69, wherein the CTL inducing peptide is HBc18-27 (875.15).

71. The pharmaceutical composition of claim 69, wherein the T helper hepatitis B core peptide selected from the group consisting of (HBc_{1-20}), (HBc_{50-69}) and ($HBc_{111-125}$).

72. The pharmaceutical composition of claim 69, wherein the lipid is linked to the N terminus of the T helper

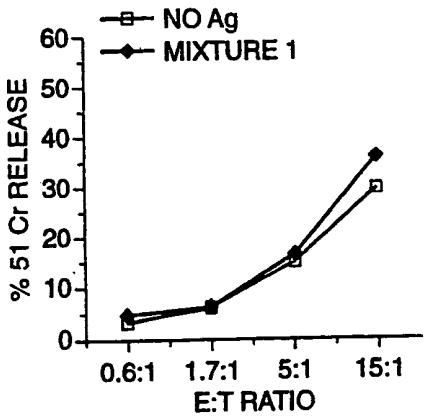

FIG. 1a

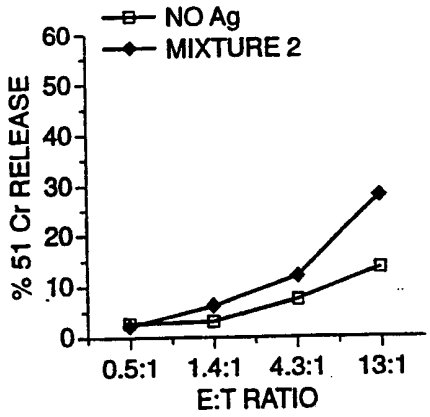

FIG. 1b

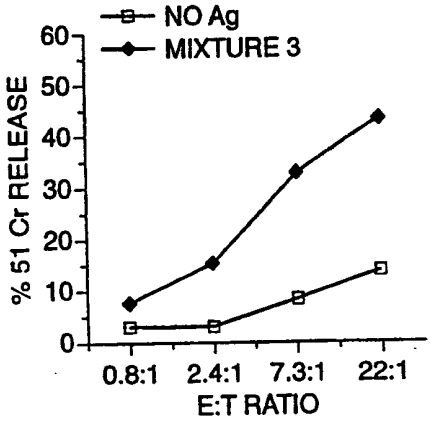

FIG. 1c

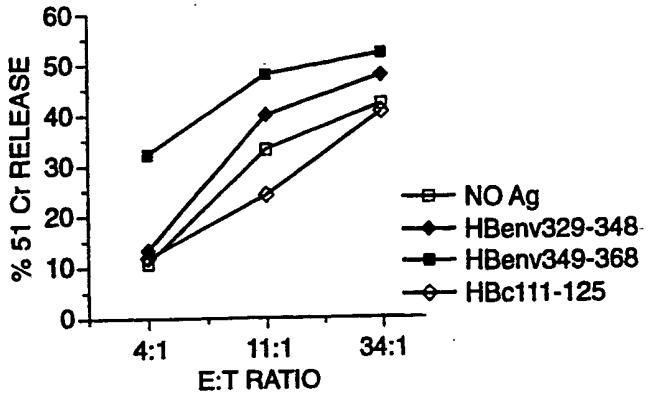

FIG. 1g

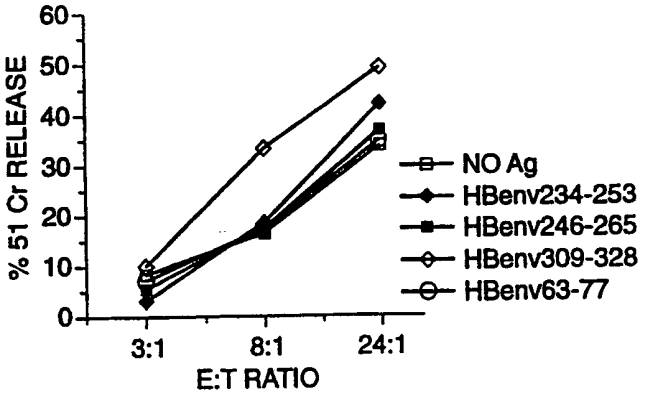

FIG. 1h

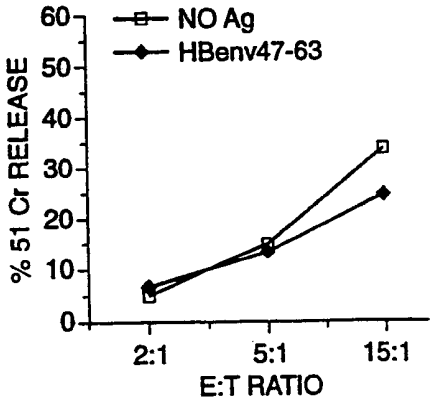

FIG. 2i

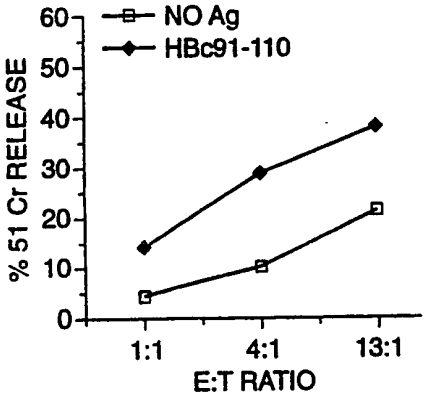

FIG. 2m

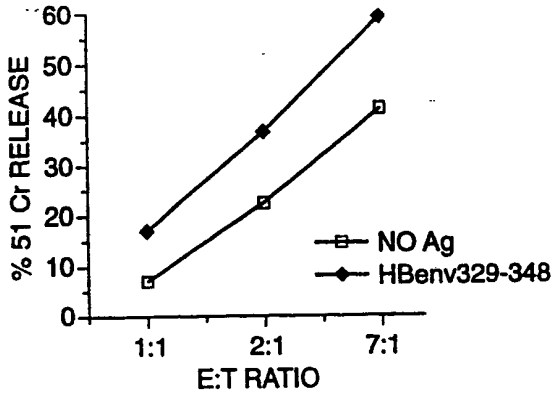

FIG. 2n

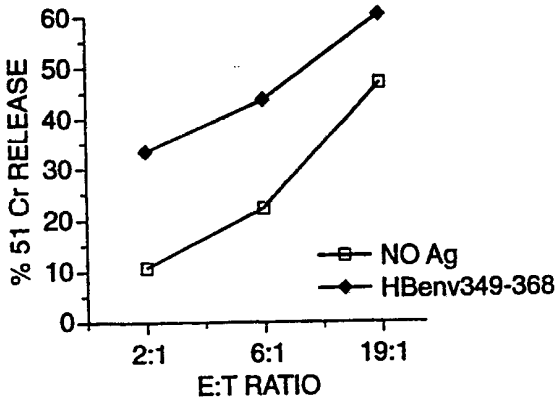

FIG. 2o

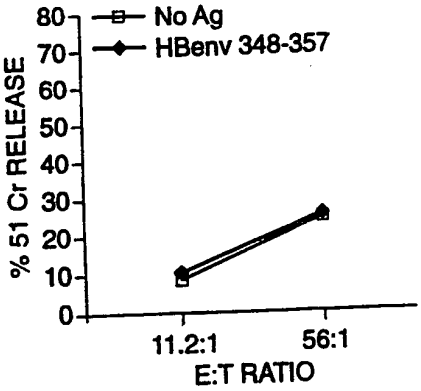

FIG. 3c

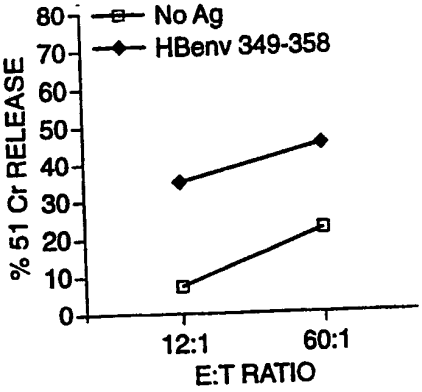

FIG. 3d

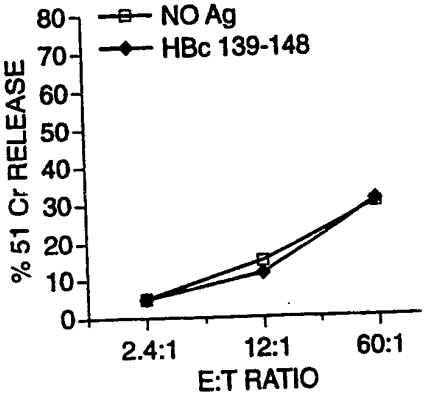

FIG. 3e

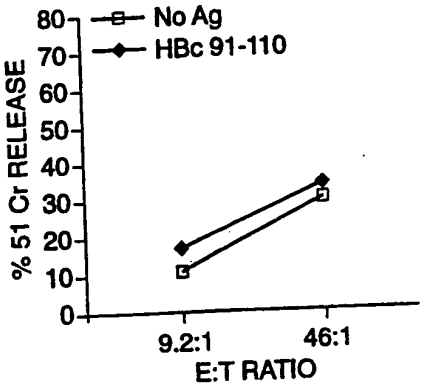

FIG. 3i

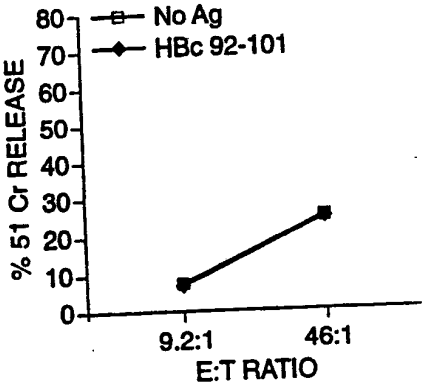

FIG. 3j

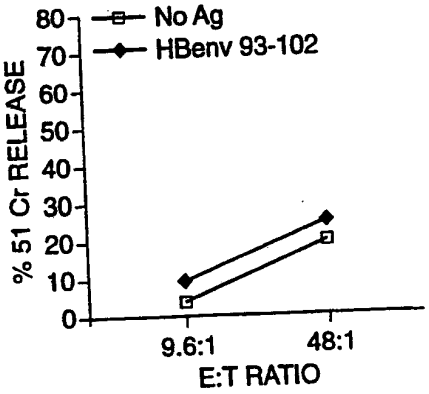

FIG. 3k

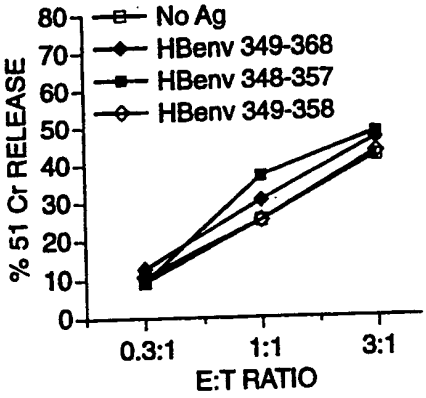

FIG. 4c

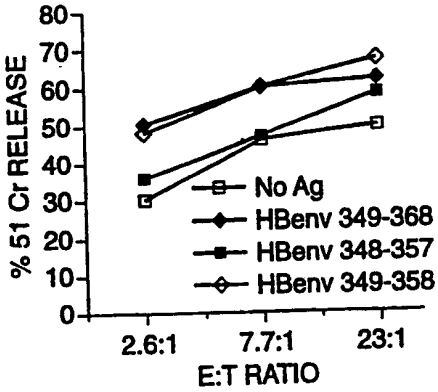

FIG. 4d

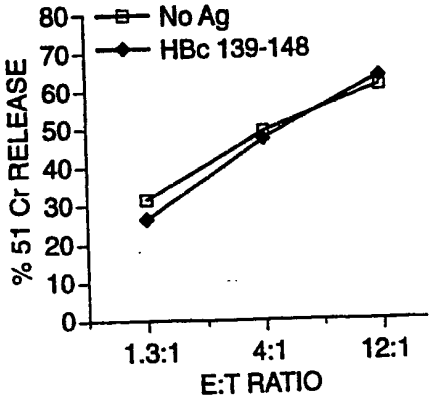

FIG. 4e

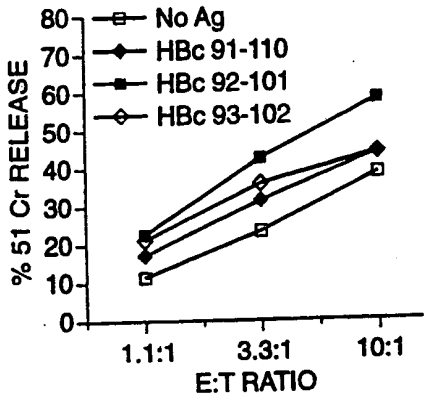

FIG. 4i

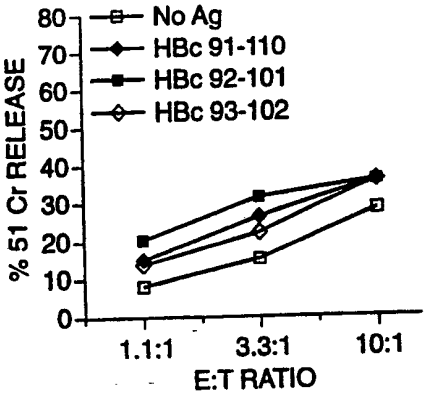

FIG. 4j

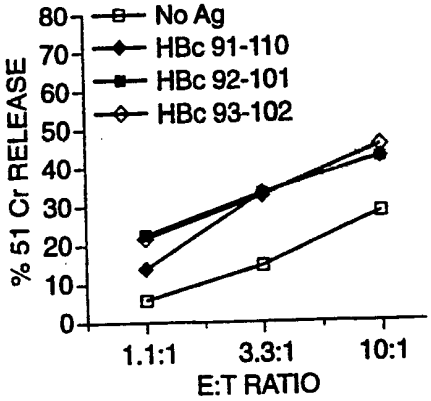

FIG. 4k

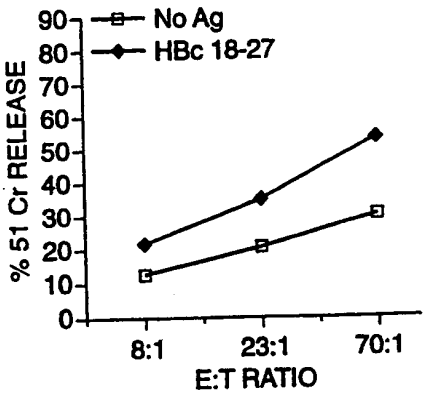

FIG. 7

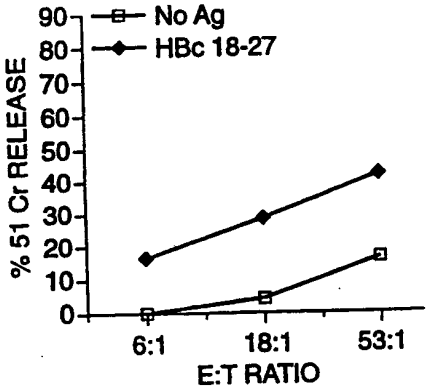

FIG. 8

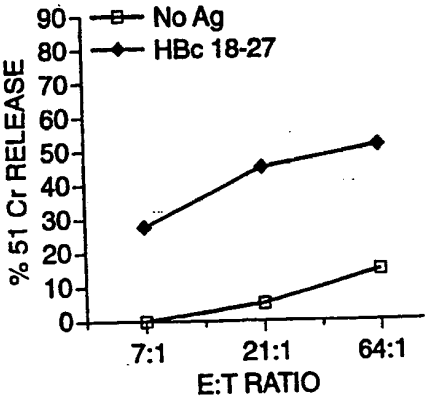

FIG. 9

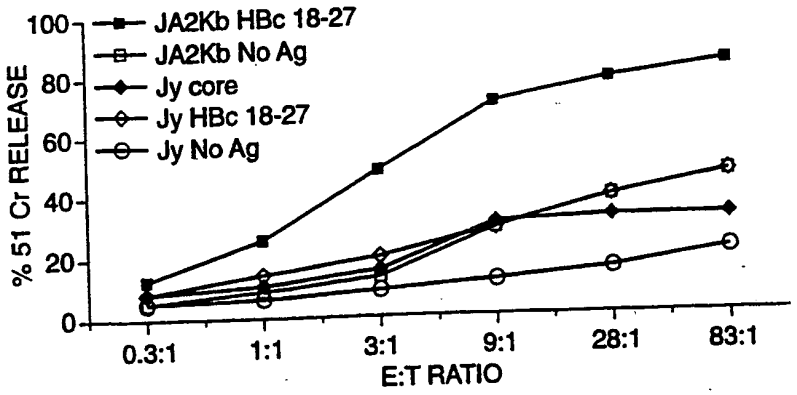

FIG. 12

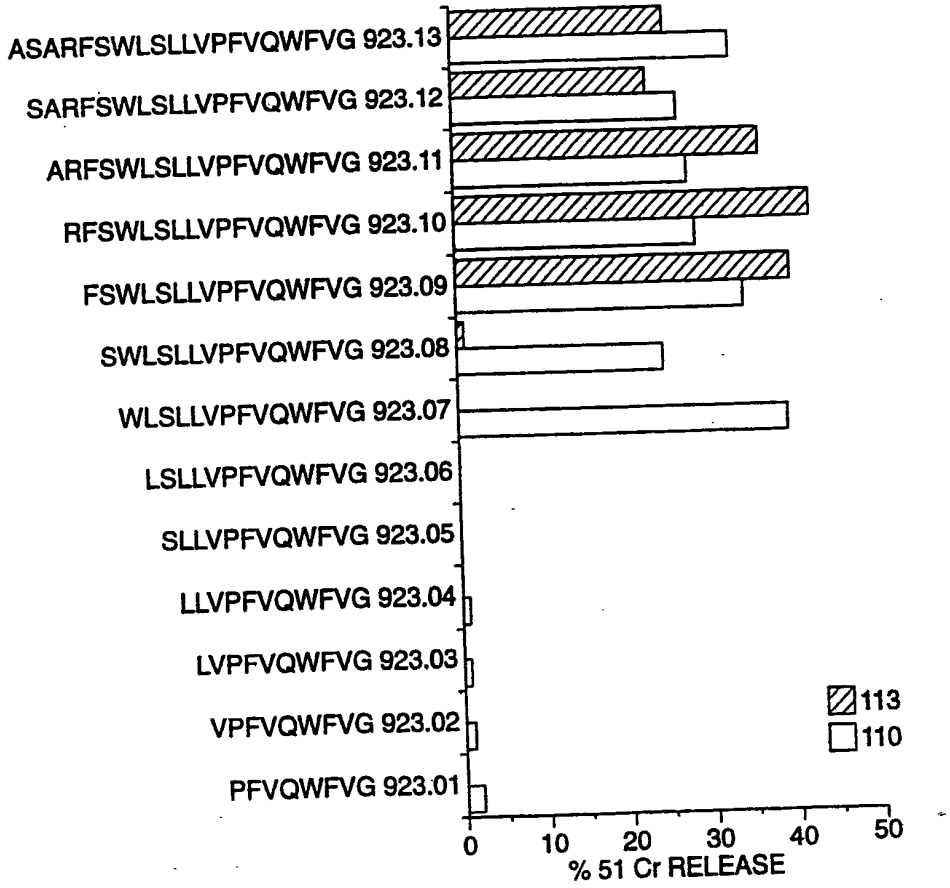

FIG. 13a

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07218

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Mannual patent search

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, APS, REGISTRY, IntelliGenetics data bases

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	The Journal of Immunology, Volume 140, No. 6, issued 15 March 1988, E. Celis et al, "Recognition of hepatitis B surface antigen by human T lymphocytes", pages 1808-1815, see pages 1808-1810.	1, 33, 59
X Y	Develop. Biol. Standard., Volume 54, issued 1983, A. R. Neurath et al, "Specificity of antibodies elicited by a synthetic peptide having a sequence in common with a fragment of a virus protein—the hepatitis B surface antigen", pages 103-112, see pages 103-105.	33, 48, 59 47, 49, 51-54
X Y	Chem. Pharm. Bull., Volume 36, No. 12, issued 1988, Y. Hayashi et al, "Studies on peptides. CLXVI. Solid-phase syntheses and immunological properties of fragment peptides related to human hepatitis B virus surface antigen (HBsAg) and its Pre-S2 gene", pages 4993-4999, see pages 4993-4995, 4997.	1, 33, 59 47-49, 51-54
X Y	US, A, 4,818,527 (Thornton et al) 04 April 1989, col. 10-12, 14, 15.	9, 15, 33, 34, 41-43, 45, 48, 59, 75

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 OCTOBER 1992	18 NOV 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer KAY K. KIM, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07218

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 39/29, 39/00, 39/385, 47/44, 47/48, 9/127; C07K 7/06, 7/08, 7/10, 7/02, 17/02, 17/06

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/88, 89, 450; 530/324, 325, 326, 327, 328, 329, 345, 359, 404, 405, 408, 409; 514/12, 13, 14, 15, 16, 17; 930/223

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

424/88, 89, 450; 530/324, 325, 326, 327, 328, 329, 345, 359, 404, 405, 408, 409, 806, 807; 514/12, 13, 14, 15, 16, 17; 930/223; 436/823, 820, 829

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I. Claims 1-55 and 59-78 drawn to CTL peptides, CTL/T conjugates, CTL/T/lipid conjugates, compositions and method of using thereof, classified in Class/subclass 530/324+, 424/88+ and 435/69.1+.

Species 1. CTL peptides of claims 1-4, including HBenv 349-368;
Species 2. CTL peptides of claims 5-8, including HBenv 329-348;
Species 3. CTL peptide of claim 10, HBenv 323-348;
Species 4. CTL peptide of claim 14, HBC 18-27;
Species 5. CTL peptide of claim 25, HBenv 309-328; and
Species 6. CTL peptides of claims 28-32, including HBC 91-110.

Group II. Claims 56-58, drawn to a method of identification, classified in Class/subclass 435/5+.

Species 1. peptide of claim 1;
Species 2. peptide of claim 5;
Species 3. peptide of claim 25; and
Species 4. peptide of claim 28.

Detailed Reasons for Holding Lack of Unity of Invention:

The invention Group II is distinct from the invention Group I in view of the fact that the product of Group I can be used in a materially different methods of using the product such as in therapy or diagnosis. The particular species within the Groups are distinct in view of the different amino acid sequences in the peptides.

Applicant is invited to elect additional invention Group(s) for which each additional invention Groups are $170.00. The search of all of 9 additional Groups will be $1,530.00. If Applicant does not elect to pay additional invention Group(s), Group I, claims 1-4, 9, 11, 13, 15, 16, 33-35, 37, 41-43, 45, 47-55, 59-67, 69, 71-75 and 78 will be searched to the extent the claims read on the CTL peptide species 1.